Supporting Information

Determination of N^2-hydroxymethyl-dG Adducts in Nasal Epithelium and Bone Marrow of Non-human Primates following $^{13}CD_2$-Formaldehyde Inhalation Exposure.

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Supporting information

Chemicals and Reagents: Deoxyguanosine, potassium phosphate, Tris-HCl, $MgCl_2$, acetic acid, $NaCNBH_3$, methanol, acetonitrile, phosphates and HPLC grade water were all purchased from Sigma (St. Louis, MO). 20% formaldehyde in water was procured from Tousimis (Rockville, MD). DNase I, alkaline phosphatase and phosphodiesterases were purchased from Sigma (St. Louis, MO). [$^{13}CD_2$]-paraformaldehyde and [$^{13}C_{10}{}^{15}N_5$]-dG was ordered from Cambridge Isotope Lab (Cambridge, MA). N^2-CH_3-dG was obtained from Berry & Associates (Dexter, MI). All chemicals were used as received unless otherwise stated. The internal standard, [$^{13}C_{10}{}^{15}N_5$]-N^2-CH_3-dG was previously prepared as described in Lu et al (11).

Animal Exposures:
Animals: Eight male, Cynomolgus macaques were culled from the Lovelace Respiratory Research Institute (LRRI) colony for this study. Animals were approximately 6 years of age at the time of exposure and weighed between 4.48-8.56 kg. Animals were conditioned to whole body exposure chambers 4 times at 30, 60, 180, and 360 minutes prior to the first day of exposure. All protocols were approved by the LRRI animal care and use committee.

Exposure: Animals received two consecutive days of whole-body inhalation exposure to [$^{13}CD_2$]-formaldehyde for 6 hours. Target exposure concentrations were 2 and 6 ppm. Prior to exposures, chamber homogeneity was confirmed to be < 5 % relative standard deviation among sampling ports. Time to 90 % of vapor concentration was <10 minutes. Oxygen levels were monitored throughout the exposure. Deuterated/^{13}C labeled formaldehyde with ≥ 98% purity was liberated by heating [$^{13}CD_2$]-paraformaldehyde. The mixture generated was directed into a Tedlar bag. Tedlar bags were filled for each exposure and changed out approximately every hour. The Tedlar bags were connected to the whole-body chamber by a stainless steel line. During the 6 hour exposures, Tedlar bags (258-1095 ppm) were directed into the whole body chamber through a supply pump running a total flow of ~ 1.5 L/min with chamber exhaust flows of up to 310 L/min. Chamber concentrations were monitored by collecting exhaust through a Waters XpoSure Aldehyde Sampler cartridge at 0.382-394 L/min. Cartridges were collected every 5 minutes continuously throughout the 6-hour exposure. Cartridges were extracted real time by passing acetonitrile (2 mL) through the cartridge and collecting extract into a beaker. Air was passed over the extracted cartridge to collect any remaining fluid. Samples were transferred to an autosampler vial and analyzed by UV-HPLC as previously described (12). Actual exposure concentrations as determined by UV-HPLC are shown in table S1.

Necropsy: Animals were sedated with Ketamine (10 mg/kg, IM) and administered Euthasol (>1ml/4.5 kg, IV). Animals were serially euthanized post exposure beginning approximately 15 minutes after the cessation of the second whole body inhalation exposure. One animal underwent necropsy at a time and each necropsy took approximately 45 minutes. All samples were collected within 3 hours of exposure. During necropsy, tissues were collected, flash frozen in liquid nitrogen, placed in individually wrapped vials, and stored at -70° to -90°C until analysis. Samples were shipped by overnight courier on dry ice to the University of North Carolina – Chapel Hill for DNA adduct measurements.

Adduct Isolation and Instrumental Analysis:
DNA Isolation and Digestion: DNA was isolated from primate tissues using a NucleoBond DNA Isolation Kit (Bethlehem, PA) as previously described by Lu et al (2010). Briefly, tissues were homogenized on ice, digested with proteinase K for 2 hours at 50°C, followed by DNA isolation using an anion exchange column. DNA was eluted, pelleted using isopropanol and centrifuged at 4°C for 15 min. The isolated DNA was dissolved in water and frozen at -80°C for later analysis. Between 10 – 390 µg of DNA were used for analysis as previously described by Lu et al (2010). Briefly, DNA was incubated with 50 mM $NaCNBH_3$ at 37°C for 6 hours in phosphate buffer (pH=7.2). This was followed by the addition of the internal standard (20 fmol) and digestion with DNase I (200U) for 10 min in the digestion buffer (80 mM Tris-HCl, 20 mM $MgCl_2$, pH=7.2), followed by the addition of 25 µl of alkaline phosphatase and 25 µl of phosphodiesterases for an additional hour. Enzymes and undigested DNA were removed by a Millipore Microcon YM-10 spin column and the resultant solution was separated by HPLC to collect the fractions containing N^2-methyl-dG adducts.

High Performance Liquid Chromatography (HPLC). The purification of formaldehyde-DNA adducts was carried out on an Agilent 1200 series HPLC system equipped with a diode-array detector (Santa Clara, CA). Analytes were separated by reverse phase chromatography using a Waters C18 T3 (150 mm × 4.6 mm, Milford, MA) analytical column. The mobile phases were 10mM ammonium acetate with 0.1% acetic acid (A) and methanol (B). The flow rate was set at 1 mL/min and a linear gradient with the following conditions was used: 0 min, 5% solvent B; 5 min, 5% solvent B; 10 min, 8% solvent B; 20 min, 10% solvent B; 30 min, 15% solvent B; 45 min, 30% solvent B; 45.1 min, 5% solvent B. 60 min, 5% solvent B. N^2-Me-dG eluted with a retention time of 26.5 min. The detector was set to 254 nM and used to quantitate dG from digested samples.

Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS). LC-MS/MS analyses were performed on a triple-stage quadrupole mass spectrometer TSQ-Quantum Ultra (Thermo Scientific, Waltham, MA) operating in selected reaction monitoring (SRM) mode to detect and quantify N^2-methyl-dG adducts along with the internal standard as previously described by Lu et al (2010b). A Waters (Milford, MA) nano-Ultra Performance Liquid Chromatography (nano-UPLC) system was used to introduce samples to the mass spectrometer. Mobile phases were comprised of water with 0.1% Acetic Acid (A) or Acetonitrile with 0.1% Acetic Acid (B). Analytes were first retained on a trap column with a flow rate of 5 µL/min of 2% mobile phase B, and subsequently transferred to the analytical column with an initial starting condition of 2% B at 0.6 µL/min for 1 minute followed by a linear gradient to 60%B over 14 min. The column was then cleaned at 80% B for 1.5 min followed by re-equilibration for an additional 7.5 min. The analytes were introduced to the MS using positive mode electrospray ionization with a source voltage of 2200 V and no additional gasses. The ion transfer tube was held at 325ºC and skimmer offset set to zero. Scan speed was set at 0.1 sec, scan width at 1.0 m/z, and peak widths for Q1 and Q3 at 0.3 and 0.5 m/z, respectively. Collision energy was set at 17eV with Argon as the collision gas set at 1.5 arb units. N^2-hydroxymethyl-dG was quantified as N^2-methyl-dG following reduction using the transition of m/z 282.2 → 166.1 and N^2-[$^{13}CD_2$]-hydroxymethyl-dG was quantified as N^2-[$^{13}CD_2$]-methyl-dG with the transition of m/z 285.2 → 169.1. Two additional transitions including m/z 284.2→ 168.1 and m/z 283.2 → 167.1 were also monitored for potential H-D exchange.

Method Validation: The method was validated using calf thymus DNA to determine accuracy, precision and recovery. N^2-methyl-dG was added to calf thymus DNA at 4, 8, 16 fmol and digested, separated by HPLC and quantified using nano-UPLC-MS/MS. For recovery experiments, calf thymus DNA was treated as described above, but with the addition of N^2-methyl-dG occurring after HPLC fraction separation. Accuracy, precision and recovery data were similar to the previously reported capillary-UPLC-MS/MS method (11) and are shown in Table S2. Quantitation was accomplished using the ratio of analyte peak area to internal standard peak area and amount of injected analytical standard along with the internal standard. Linear calibration curves were generated with r^2>0.98 and used for the quantitation of the analytes. The method had a limit of detection of 20 amol on column, as shown in figure S1. The limit of quantitation was 40 amol on column.

Exposure Day	Target Concentration (ppm)	Chamber Concentration (ppm)
Exposure Day 1	2.0	1.8 ± 0.4
Exposure Day 2	2.0	2.1 ± 0.3
Average	2.0	1.9
Exposure Day 1	6.0	6.1 ± 1.6
Exposure Day 2	6.0	6.0 ± 1.2
Average	6.0	6.1

Table S1 – Exposure Concentrations. $^{13}CD_2$-formaldehyde exposure concentrations were targeted at 2 and 6 ppm for two consecutive days. The actual inhalation chamber concentrations were monitored and are shown above.

Expected Concentration (fmol)	Calculated Concentration (fmol)	% Accuracy	Precision (%CV)	% Recovery
4.0	3.7	91	12	52
8.0	6.2	77	16	39
16	14	87	6.1	59
Average		85	11	50

Table S2 – Method Validation. Calf thymus DNA was fortified with 4, 8, and 16 fmol of N^2-methyl-dG both pre- and post-processing (n = 3 per level) to determine the average precision, accuracy and recovery.

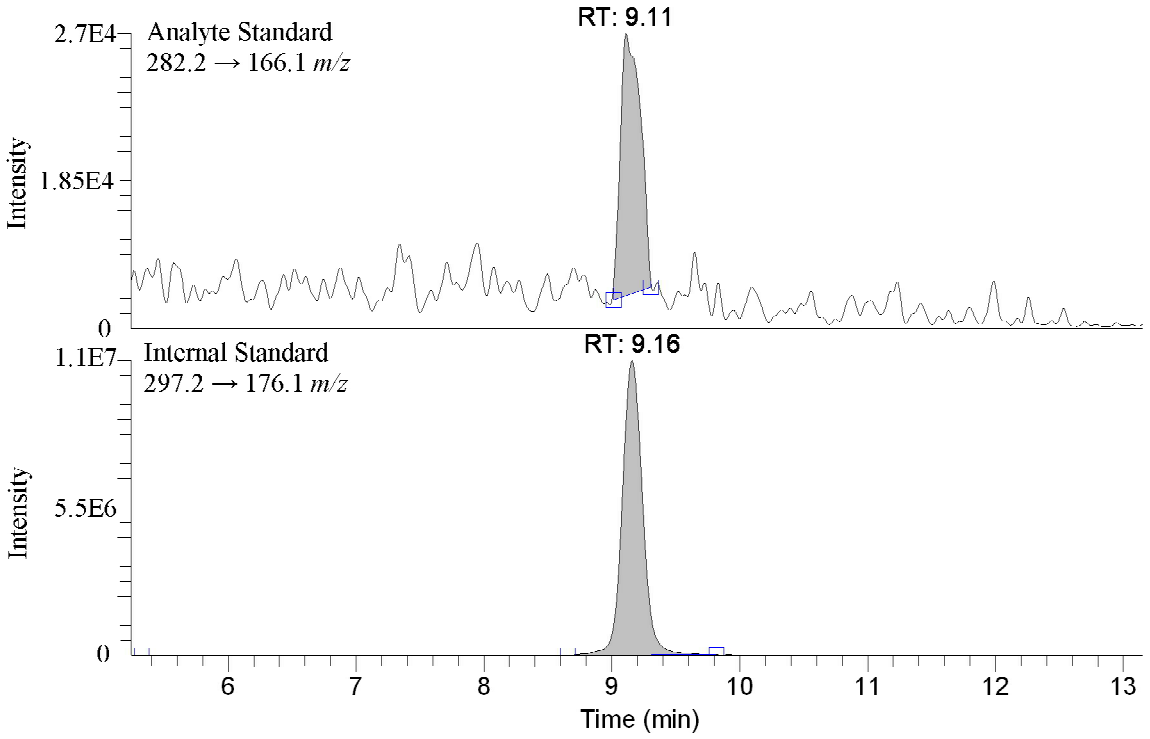

Figure S1 – Limit of Detection. Two chromatograms showing the limit of detection (20 amol on column) for N^2-methyl-dG, and the internal standard ($^{15}N_5{}^{13}C_{10}$-methyl-dG) are shown.